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DELLA proteins interact with FLC to repress flowering transition

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Abstract Flowering is a highly orchestrated and extremely critical process in a plant's life cycle. Previous study has demonstrated that SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and FLOWERING LOCUS T (FT) integrate the gibberellic acid (GA) signaling pathway and vernalization pathway in regulating flowering time, but detailed molecular mechanisms remain largely unclear. In GA signaling pathway, DELLA proteins are a group of master transcriptional regulators, while in vernalization pathway FLOWERING LOCUS C (FLC) is a core transcriptional repressor that down-regulates the expression of *SOC1* and *FT*. Here, we report that DELLA proteins interact with FLC *in vitro* and *in vivo*, and the LHRI domains of DELLAs and the C-terminus of MADS domain of FLC are required for these interactions. Phenotypic and gene expression analysis showed that mutation of *FLC* reduces while over-expression of *FLC* enhances the GA response in the flowering process. Further, DELLA-FLC interactions promote the repression ability of FLC on its target genes. In summary, these findings report that the interaction between MADS box transcription factor FLC and GRAS domain regulator DELLAs may integrate various signaling inputs in flowering time control, and shed new light on the regulatory mechanism both for FLC and DELLAs in regulating gene expression.

Keywords: *Arabidopsis*; co-repressor; DELLAs; FLC; flowering
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INTRODUCTION

Precise transition from vegetative to reproductive development is crucial for reproductive success in flowering plants. It is tightly regulated by endogenous developmental cues and exogenous environmental stimulus. In *Arabidopsis*, extensive molecular genetic analyses have elucidated five flowering pathways, including the photoperiod, autonomous, vernalization, gibberellic acid (GA) and age pathways (Michaels 2009; Amasino 2010; Srikanth and Schmid 2011). FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) are two major floral regulators that integrate both developmental and environmental cues into the floral genetic networks. They further activate a series of genes involved in the formation of floral meristems (Abe et al. 2005; Moon et al. 2005; Teper-Bamnolker and Samach 2005; Wigge et al. 2005).

FLOWERING LOCUS C (FLC) is another crucial floral regulator that integrates autonomous and vernalization pathways to fine-tune flowering time. *FLC* encodes a MADS-box transcription factor, which belongs to the family containing five other MADS AFFECTING FLOWERING (MAF) proteins (Michaels and Amasino 1999; Sheldon et al. 1999; De Bodt et al. 2003; Ratcliffe et al. 2003). FLC acts as a strong repressor of floral transition by direct binding to the promoters of several flowering time genes (*SOC1* and *FT*) and repressing their transcription. The expression level of *FLC* quantitatively and reversely correlates with flowering time (Michaels and Amasino 1999; Sheldon et al. 1999). There are several regulatory pathways that converge to regulate FLC: the FRIGIDA pathway activates *FLC* expression, the autonomous pathway down regulates *FLC*, and vernalization epigenetically silences *FLC* in response to prolonged cold. All of these pathways involve a set of antisense transcripts, collectively named as COOLAIR (Swiezewski et al. 2009; Hornyik et al. 2010; Liu et al. 2010) and COLDAIR (Heo and Sung 2011; Hu et al. 2014). Epigenetic regulation of *FLC* by proteins and long noncoding RNAs in transcription levels have been extensively studied (Bastow et al. 2004; Sung and Amasino 2004; Margueron et al. 2005; Sung et al. 2006; Schmitz and Amasino 2007; Choi et al. 2011; Heo and Sung 2011; Jeon and Kim 2011; Kim and Sung 2012). But whether FLC could be modulated by other mechanisms is still unclear. Recent ChIP-seq results showed that FLC can bind to many other genes, indicating that FLC has other regulatory roles in addition to repression of flowering (Deng et al. 2011). More interestingly, the active form of FLC is a high molecular complex of approximately 800 kDa (Helliwell et al. 2006), indicating that more members probably take part in this complicated process by physically interacting with FLC.

GAs are essential for floral induction both in short-day, long-day and continuous light conditions in *Arabidopsis* (Wilson et al. 1992; Silverstone et al. 1997; Blazquez et al. 1998; Griffiths et al. 2006). GAs function through overcoming the repressive effects of the DELLA proteins (Richards et al. 2001). DELLA proteins including REPRESSOR OF GA (RGA), GA INSENSITIVE (GAI), RGA-LIKE1 (RGL1), RGL2 and RGL3, are nuclear localized transcriptional regulators that play pivotal

roles in inhibiting GA responses (Sun and Gubler 2004). Bioactive GA binds to its receptor GA INSENSITIVE DWARF1 (GID1) and enhances the interaction between GID1 and DELLAs. This interaction induces the rapid degradation of DELLA proteins via the ubiquitin-proteasome pathway (Silverstone et al. 2001; Ueguchi-Tanaka et al. 2005; Griffiths et al. 2006; Willige et al. 2007). E3 ubiquitin ligase $SCF^{SLY1/GID2}$ promotes polyubiquitination of DELLAs in this process (McGinnis et al. 2003; Sasaki et al. 2003; Dill et al. 2004; Fu et al. 2004). GAs promote flowering through the activation of genes encoding the floral integrator SOC1, LEAFY (LFY) and FT (Mutasa-Gottgens and Hedden 2009). A recent study showed that the five DELLA proteins can repress flowering (Galvao et al. 2012). MiRNA159, which was negatively regulated by DELLA, represses *MYB DOMAIN PROTEIN33* (*MYB33*) and thus controls the expression of *LFY* (Achard et al. 2004). Further, it was reported that DELLAs interact with SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) to interfere with SPL transcriptional activity and affect flowering time (Yu et al. 2012).

Given that DELLA proteins possess no canonical DNA-binding domain, it seems that DELLAs modulate gene expression by interacting with other transcription factors, such as PHYTOCHOME INTERACTING FACTORs (PIFs), SCARECROW-LIKE3 (SCL3), MYC2, ALCATRAZ (ALC), ETHYLENE INSENSITIVE3 (EIN3), BRASSINOZALE-RESISTANT1 (BZR1) SPLs (Zentella et al. 2007; de Lucas et al. 2008; Feng et al. 2008; Arnaud et al. 2010; Hou et al. 2010; Zhang et al. 2011; An et al. 2012; Bai et al. 2012; Hong et al. 2012; Yang et al. 2012; Yu et al. 2012). Several mechanisms about DELLA-mediated transcriptional control have been demonstrated (Gao et al. 2011; Locascio et al. 2013). One is that DELLAs act as repressors by interacting with the DNA-binding domain of transcription factors mentioned above and inhibiting their DNA-binding abilities. Another one is that DELLAs interact with a negative regulator (such as JASMONATE ZIM-DOMAIN (JAZ)) to release its inhibition on the transcription factor (Hou et al. 2010). Recent studies also demonstrated that DELLAs interact with transcription factors (Such as BOTRYTIS SUSCEPTIBLE1 INTERACTOR (BOI), GAI-ASSOCIATED FACTOR1 (GAF1), ABA-INSENSITIVE3 (ABI3), ABA-INSENSITIVE5 (ABI5), INDETERMINATE DOMAIN1 (IDD1), DELLA INTERACTING PROTEIN 1 (DIP1)) and function coincidently with transcription factors to activate target gene expression (Lim et al. 2013; Park et al. 2013; Fukazawa et al. 2014; Yoshida et al. 2014; Yu et al. 2014).

Here, we reveal the molecular mechanism underlying the crosstalk between DELLAs and FLC in regulating flowering time. DELLAs regulate flowering time partially through a direct interaction with FLC, and this interaction enhances the transcriptional inhibition ability of FLC to its target genes *SOC1* and *FT*. This research not only adds a new regulatory layer on FLC, but also indicates a novel mechanism for DELLAs on regulating down-stream gene expression.

RESULTS

DELLAs interact with FLC *in vitro* and *in vivo*

DELLA proteins function as key transcriptional regulators that mediate the effects of GA on plant development. To further investigate the function of DELLA proteins, we performed a yeast two-hybrid (Y2H) experiment to identify RGA-interacting partners using an *Arabidopsis* transcription factor library (Ou et al. 2011). A truncated form of RGA with N-terminal DELLA/TVHYNP motif deleted was used as bait, since full-length DELLAs exhibited strong auto-transactivation activity in yeast. FLC protein was identified as a candidate interacting protein in Y2H. Besides RGA, FLC also interacted with other *Arabidopsis* DELLAs, namely, GAI, RGL1, RGL2 and RGL3 (Figure 1A). A fragment of EIN3 protein 200-500 amino acid residue (aa) also interacts with all DELLAs as reported previously (An et al. 2012), which was used as a positive control (Figure 1A). Direct interaction between RGA and FLC was also confirmed by pull-down assay (Figure 1B).

To further investigate DELLAs-FLC interactions in planta, bimolecular fluorescence complementation (BiFC) was performed in *Arabidopsis* suspension cells. Reconstituted yellow fluorescent signals, caused by the interaction of FLC-nYFP (FLC fused with N-terminal part of YFP) and RGA-cYFP/GAI-cYFP (RGA or GAI fused with C-terminal part of YFP), were observed in the nuclei. No evident fluorescent signals were found in FLC-nYFP co-transfected with cYFP and RGA-cYFP, GAI-cYFP, or cYFP co-transfected with nYFP. Only weak background signal with less frequency and weak intensity was observed in cells co-expressing nYFP and cYFP-GAI (Figure 1C). These results suggest that FLC binds to RGA/GAI in the nuclei of *Arabidopsis* cells. In addition, co-immunoprecipitation (Co-IP) studies using transgenic line *FLCox/TAP-RGA* further confirmed this interaction in planta (Figure 1D). Together, these results suggest that FLC is a new DELLA-associated transcription factor.

The C-terminus of MADS domain of FLC and the LHRI domain of RGA are required for the interaction between FLC and RGA

To determine which domains of FLC and RGA proteins are important for their interaction, we did a series of Y2H assays. FLC is a type II MADS family protein harboring MADS-domain (M), intervening region (I), keratin-BOX (K) and C-terminal domain (C) (Riechmann and Meyerowitz 1997b, a; De Bodt et al. 2003). Several overlapping fragments of FLC were cloned into pGADT7 vector to examine their potential interaction with DELLAs (Figure 2A). FLC 1-93 aa, 1-58 aa and 39-78 aa could interact with DELLAs, suggesting FLC 39-58 aa is sufficient for the interaction (Figure 2B). Deleting FLC 39-58 aa (FLC-△MADS) abolished the interaction with DELLAs, indicating that FLC 39-58 aa is necessary for the DELLA and FLC interaction (Figure 2C). FLC 58-93 aa with RGA-M5/GAI-M5 and FLC-△MADS with RGA-M5/GAI-M5 were detected by western blot (Figure S1A).

DELLAs belong to a subclass of the plant-specific GRAS family. The N-terminus of DELLA contains two conserved domains, DELLA domain and TVHYNP domain. The C-terminus of DELLA proteins is involved in transcriptional regulation and is composed of two Leu heptad repeats (LHRI and LHRII) and three conserved motifs, VHIID, PFYRE and SAW (Itoh et al. 2002; Ueguchi-Tanaka et al. 2007). Previous study has shown that the LHRI domain of RGA was essential for its activity *in vivo* and mediated the interactions between RGA and other transcription factors, such as PIFs, JAZ1 and BZR1 (de Lucas et al. 2008; Hou et al. 2010; Bai et al. 2012; Li et al. 2012). To determine whether LHRI domain is also important for

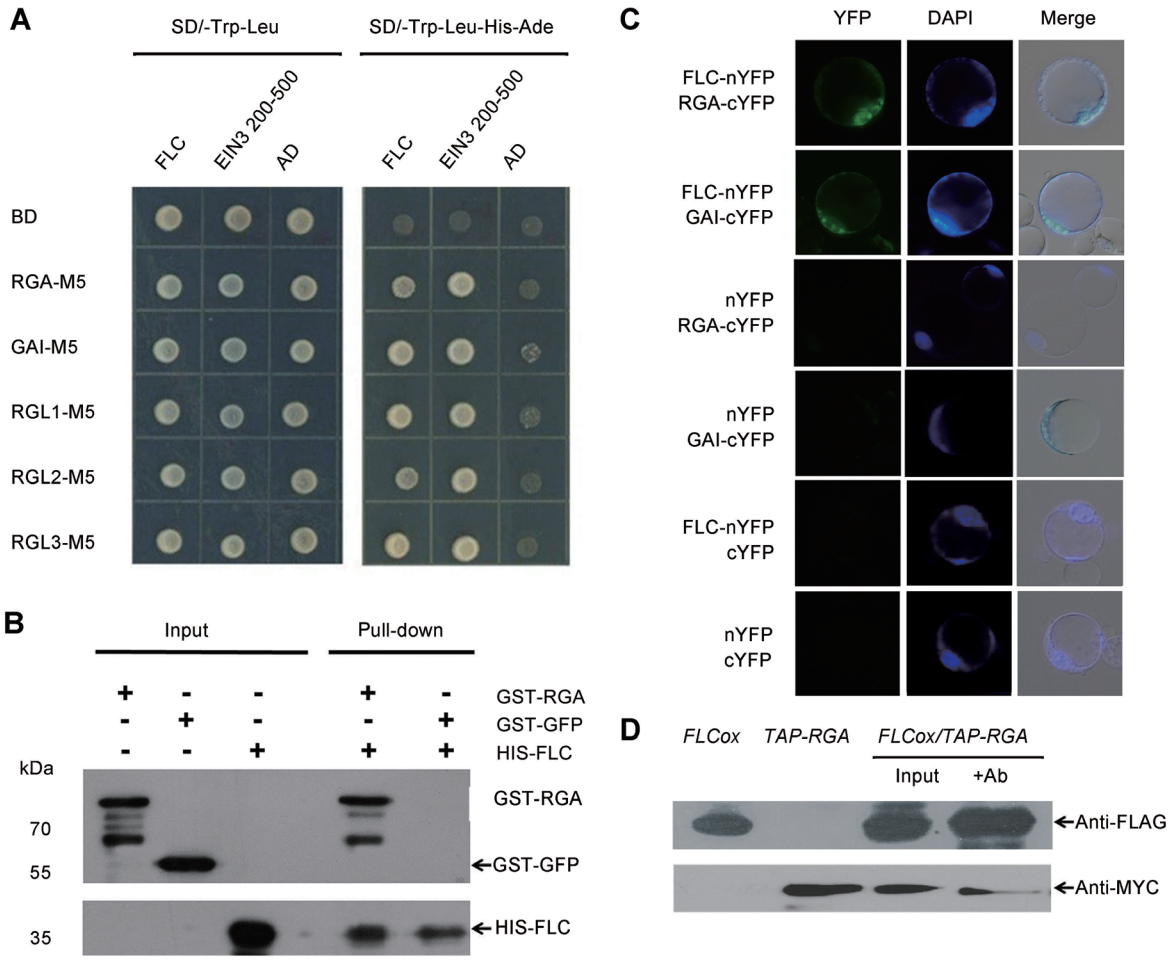

Figure 1. DELLAs interact with FLC *in vitro* and *in vivo*

(A) Interaction between DELLAs and FLC protein in yeast. DELLA proteins without self-activation domain (RGA-M5 209-587 aa, GAI-M5 158-533 aa, RGL1-M5 140-512 aa, RGL2-M5 168-548 aa, RGL3-M5 147-524 aa) were constructed into pGBKT7 separately and full-length FLC was constructed into pGADT7. The interaction of DELLAs and EIN3 200-500 aa was used as positive controls. **(B)** *In vitro* pull-down assay for RGA and FLC interaction. The interaction was determined by western blot using GST antibody. GST-GFP fusion protein was used as a negative control. **(C)** BiFC analysis of RGA/GAI and FLC interaction *in planta*. YFP fluorescence was detected 12 h after coexpression of the indicated construct pairs in *Arabidopsis* suspension cells. The nuclei are indicated by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. **(D)** Co-IP assays showing RGA association with FLC in *Arabidopsis*. Eight-d-old *FLCox*/*TAP-RGA* T2 seedlings treated with 10 μmol/L MG132 for 4 h were used for Co-IP. FLAG tag was fused with FLC protein and TAP, containing nine repeats of MYC epitope, was fused to RGA protein. Proteins were immunoprecipitated with anti-FLAG M2 agarose beads (+Ab) and detected with either anti-MYC or anti-FLAG antibodies.

interaction with FLC, truncated RGA were cloned into pGBKT7 vector to examine their potential interactions with full-length or 1-93 aa of FLC (Figure 3A). The fragment RGA-M5 (209-588 aa) and RGA-M5△SAW (209-506 aa) could interact with FLC and FLC 1-93 aa in Y2H, whereas RGA-M5△LHRI (265-586 aa) could not (Figure 3B), although both RGA- M5△LHRI and FLC 1-93 aa proteins can be detected by western blot (Figure S1B), indicating the LHRI domain of RGA is required for the interaction between FLC and RGA.

Therefore, we conclude that the C-terminus of MADS domain (39-58 aa) of FLC and the LHRI domain (209-506 aa) of RGA are responsible for the interaction between these two proteins.

RGA promotes the repression ability of FLC

We used chromatin immunoprecipitation assay followed by quantitative real-time PCR (ChIP-qPCR) to examine how the interaction between FLC and DELLAs affects the function of FLC in gene regulation. Previous studies showed that DELLAs not only repress several transcriptional activators, including PIFs, MYC2, BZR1 and EIN3, by weakening their DNA binding abilities (An et al. 2012; Hong et al. 2012; Xiang et al. 2012), but also interfere with several components of hormonal and developmental signaling pathways through protein–protein interaction (Hou et al. 2010; Zhang et al. 2011; Qi et al. 2014). In other cases, DELLAs were also identified as coactivators, for instance by interacting with IDDs, GAF1 transcription factors

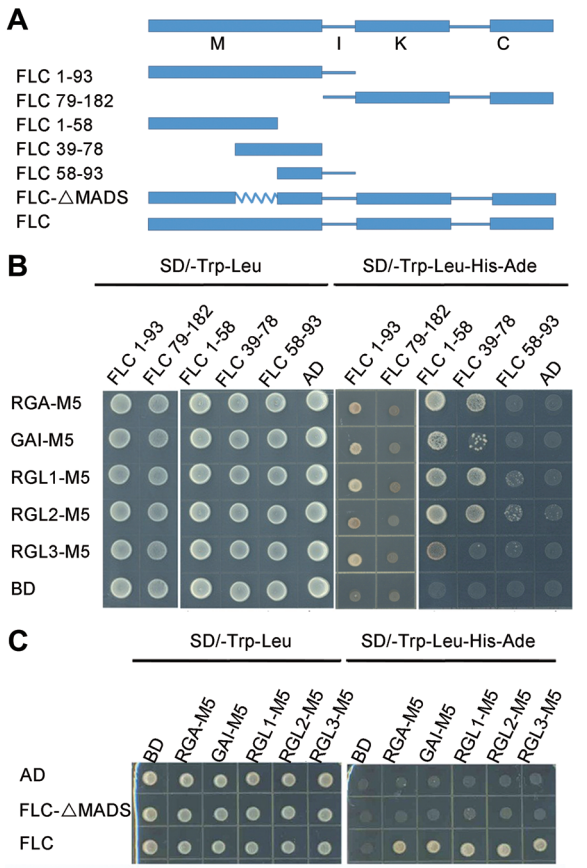

Figure 2. C-terminus of MADS domain in FLC is necessary for DELLA binding

(A) FLC fragments used in yeast two-hybrid assay. FLC contains MADS-domain (M), intervening region (I), keratin-BOX (K) and C-terminal domain (C). **(B)** The C-terminus of MADS-domain in FLC interacts with RGA-M5 and GAI-M5 in yeast. Empty vectors of BD and AD were used as negative controls. FLC 58-93 aa and RGA-M5, GAI-M5 proteins were produced when they were co-transferred separately into yeast as showed by western bolt in Supplement Figure S1A. **(C)** FLC△39-58 does not interact with DELLAs in yeast. The full-length of FLC was used as positive controls. FLC-△MADS and RGA-M5, GAI-M5 proteins were produced when they were co-transferred separately into yeast as shown by western bolt in Figure S1A.

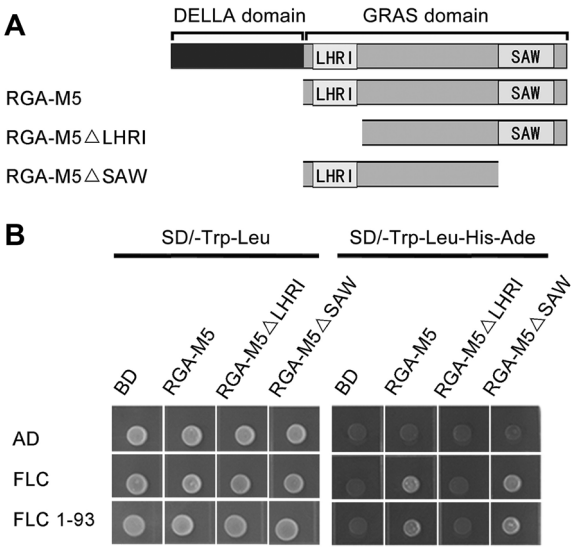

Figure 3. LHRI domain of RGA is required for FLC binding

(A) RGA fragments used in yeast two-hybrid assay. DELLA contains DELLA domain and GRAS domain. **(B)** RGA-M5 and RGA-M5△SAW interact with FLC and FLC 1-93 aa in yeast. RGA-M5 without LHRI motif (RGA-M5△LHRI) does not interact with FLC or FLC 1-93 aa. FLC 1-93 aa and RGA-M5△LHRI proteins were produced when they were co-transferred separately into yeast as shown by western bolt in Figure S1B.

(Fukazawa et al. 2014; Yoshida et al. 2014). For FLC, the N-terminus of MADS domain is mainly involved in DNA binding, and the C-terminus of MADS domain, including RGA/GAI binding fragment 39-58 aa, functions as a dimerization interface (de Folter and Angenent 2006). Based on the fact that both FLC and DELLA play negative roles in regulating plant flowering time, we proposed that interaction with DELLA proteins may enhance the transcriptional repression ability of FLC on target genes.

To explore the role of DELLAs in FLC-mediated transcription inhibition, FLC was overexpressed in transgenic plant *TAP-RGA*, and named as *FLCox/TAP-RGA*. Immunoblotting analysis using anti-FLAG antibody showed a specific FLC-FLAG protein band (Figure S2A), indicating the transgenic line was suitable for the subsequent analysis. We first examined whether RGA is associated with DNA through directly interacting with FLC. The ChIP-qPCR results showed that *in vivo* association of RGA to the transcriptional-regulation-related sequence (including promoter or intron) but not 3′ untranslation regions (3′UTRs) of FLC target genes, *SOC1* and *FT*, was increased upon FLC overexpression (Figure 4A). Because DELLAs lack a canonical DNA binding domain, these results indicate that association of DELLAs to *SOC1* and *FT* may be mediated through their interaction with FLC.

We further detected whether the binding of FLC to its target genes is affected by DELLA interaction. Two-week-old *FLCox/TAP-RGA* seedlings treated with GA or the corresponding solvent (mock) were used for ChIP-qPCR. The results showed that GA treatment decreased the binding of FLC to the *SOC1* and *FT* genes compared with mock treatment. In contrast, a fragment from the 3′UTRs did not show detectable enrichment changes (Figure 4B). These results suggest that DELLA proteins may enhance the binding ability of FLC to its target genes.

Next, transient reporter assay was conducted to study the transcriptional activity of the DELLA/FLC complex. Considering that FLC binds to the first intron of *FT*, but to the promoters of *SOC1* and *SEPALLATA3* (*SEP3*) (Deng et al. 2011), we used the upstream promoter regions of *SOC1* and *SEP3* to drive firefly luciferase (LUC) reporters in dual-luciferase assay in tobacco leaves (Figure 4C). The results showed that the expression level of luciferase driven by *SOC1* and *SEP3* promoters were markedly reduced by expression of FLC

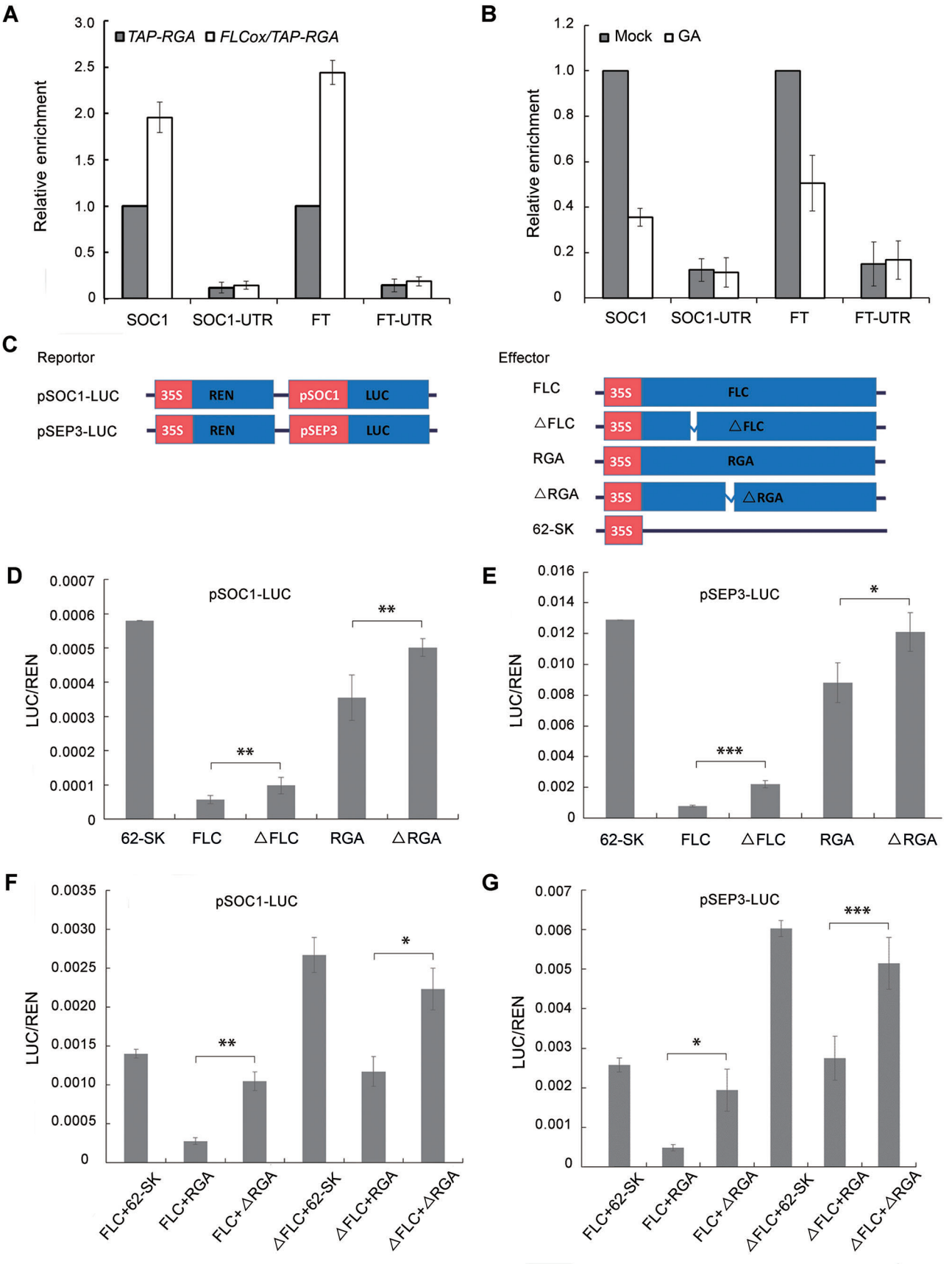

Figure 4. Continued.

(Figure 4D,E), and further suppressed by co-expression of RGA with FLC (Figure 4F,G). Expression of RGA alone was also able to repress the *SOC1* and *SEP3* promoter activities, but expression of △RGA (deletion of LHRI domain in RGA) could not (Figure 4D,E), suggesting that the LHRI domain is critical for RGA repression on FLC target genes. Notably, expression of △FLC (deletion of 39-58 aa of FLC) also led to the repression of *SOC1* and *SEP3* promoter activities, although to a lesser extent compared with expression of wild-type FLC (Figure 4D,E). Co-expression of △FLC and RGA resulted in an additional effect, but the degree of repression was much weaker than that of FLC/RGA co-expression (Figure 4F,G). Taken together, these results indicate that RGA reinforces the repression ability of FLC to target genes by interaction with it.

FLC mediates, at least partly, the effect of GA in flowering time regulation

Previous studies showed that SOC1 integrates vernalization and gibberellin signals to regulate plant flowering time, and that *soc1* null mutant was less sensitive to GA. Since *SOC1* is a direct target of FLC, and data presented above showed DELLAs interact with FLC to enhance its transcriptional repressing ability, we then examined whether FLC is an integration node for vernalization and gibberellin signals in flowering time regulation.

If FLC mediates the GA-regulated flowering process, it is expected that depleting or overexpressing *FLC* would alter the GA response in terms of floral induction. To test this possibility, we generated transgenic *Arabidopsis* plants over-expressing *FLC* fused with a FLAG tag (*35S::FLC-FLAG* or *FLCox*). Adult *FLCox* transgenic plants displayed an obvious late-flowering phenotype as shown in Figure 5A, indicating that this fusion protein was functional *in planta*. Immunoblotting analysis using anti-FLAG antibody showed specific FLC-FLAG protein band (Figure S2B), indicating the transgenic line was suitable for the subsequent analysis. The flowering-time phenotypes of *FLCox*, Col-0 and *flc-3* treated with GA or mock were analyzed. Fifty μmol/L GA or mock were sprayed each 2 d after seedlings were transferred into soils. In long-day conditions, compared with Col-0 and *FLCox* seedlings, *flc-3* seedlings treated with 50 μmol/L GA were the first ones to bolt. *FLCox* flowered later than Col-0, and were still responsive to GA. On the 20th d, with GA treatment, all of the three genotypes were bolting. At the same time point, *flc-3* and Col-0 with mock treatment showed flower buds, but *FLCox* with mock treatment did not (Figure 5A).

We next calculated the effect of depletion or over-expression of FLC on GA-elicited floral induction by determining the days to bolt (DTB) as well as the number of rosette leaves upon bolting (RLN), two commonly used flowering-time indexes. If GA-regulated flowering pathway is not affected by FLC, it is proposed that the reduction of RLN after GA application would be similar in all the three kinds of FLC-related seedlings. As shown in Figure 5, in wild-type Col-0 plants, we observed 23.7% reduction in DTB and 26.3% reduction in RLN after GA application. By contrast, in *FLCox*, GA treatment led to the reduction of DTB and RLN by 30.2% and 33.9%, respectively. On the other hand, in *flc-3*, the reduction of DTB and RLN triggered by GA application was 20.6% and 18.8%, respectively (Figure 5B–D).

We also performed similar experiments under short-day conditions. Compared with Col-0, the reduction of RLN elicited by GA application in *flc-3* was decrease from 23.7% to 16.2%, but increased from 23.7% to 26.5% in *FLCox* (Figure 5E,F).

Taken together, we conclude that *FLC*-overexpression plants are more sensitive to GA, while *flc-3* mutants are less sensitive, indicating that FLC mediates, at least partly, the effect of GA in the control of flowering time.

FLC and DELLAs regulate overlapping genomic targets including flowering related genes

We next examined whether FLC and DELLAs coordinately regulate genome-wide gene expression. Taking advantage of previously published ChIP-seq data, we found that genes affected in the absence of DELLA and genes targeted by FLC overlapped significantly (Cheminant et al. 2011; Deng et al. 2011) (Figure 6A). Among 464 direct targets of FLC identified

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Figure 4. RGA enhance the transcriptional repression activity of FLC

(A) FLC overexpression increases the level of DELLA-DNA binding *in vivo*. ChIP was performed using anti-MYC antibodies recognizing TAP-RGA followed by quantitative polymerase chain reaction (qPCR) analysis. The amount of DNA amplified from *FLCox/TAP-RGA* seedlings were normalized to that from *TAP-RGA* plants, 3'-UTR (untranslated region) fragments of SOC1 and UTR were used as negative controls. Error bars represent ± *SD*, $n = 3$. The experiment was repeated three times with similar results. **(B)** GA treatment decreases the level of FLC-DNA binding *in vivo*. ChIP was performed using anti-FLAG antibodies recognizing FLAG-FLC followed by qPCR analysis. The amount of DNA amplified from *FLCox/TAP-RGA* seedlings treat with GA normalized to that from mock, 3'-UTR fragments of SOC1 and UTR were used as negative controls. Error bars represent ± *SD*, $n = 3$. The experiment was repeated three times with similar results. **(C)** The schematic diagram shows the constructs used in the transient expression assays of **(D)** to **(G)**. The promoter of *SOC1* or *SEP3* was cloned into pGreenII 0800-LUC. FLC, △FLC (full-length FLC without the C-terminus of MADS domain), RGA and △RGA (full-length RGA without the LHRI domain) were cloned into pGreen 62-SK. **(D)** The transcriptional repression of *SOC1* promoter by FLC, △FLC, RGA and △RGA respectively in tobacco leaves. **(E)** The transcriptional repression of *SEP3* promoter by FLC, △FLC, RGA and △RGA respectively in tobacco leaves. **(F)** Effect of RGA on FLC transcriptional activity at the *SOC1* promoter reporter in tobacco leaves. **(G)** Effect of RGA on FLC transcriptional activity at the *SEP3* promoter reporter in tobacco leaves. In **(D)** to **(G)**, tobacco leaves were transformed with the dual luciferase reporter construct containing pSOC1::LUC (luciferase) and 35S::REN (renilla luciferase), and constructs overexpressing the indicated effectors. The LUC activity was normalized to REN. Transient reporter gene assays show RGA enhances the inhibition ability of FLC. Each bar in **(D)** to **(G)** represents ± *SD*, $n = 3$. Student's *t*-test was performed to determine the significance of differences ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

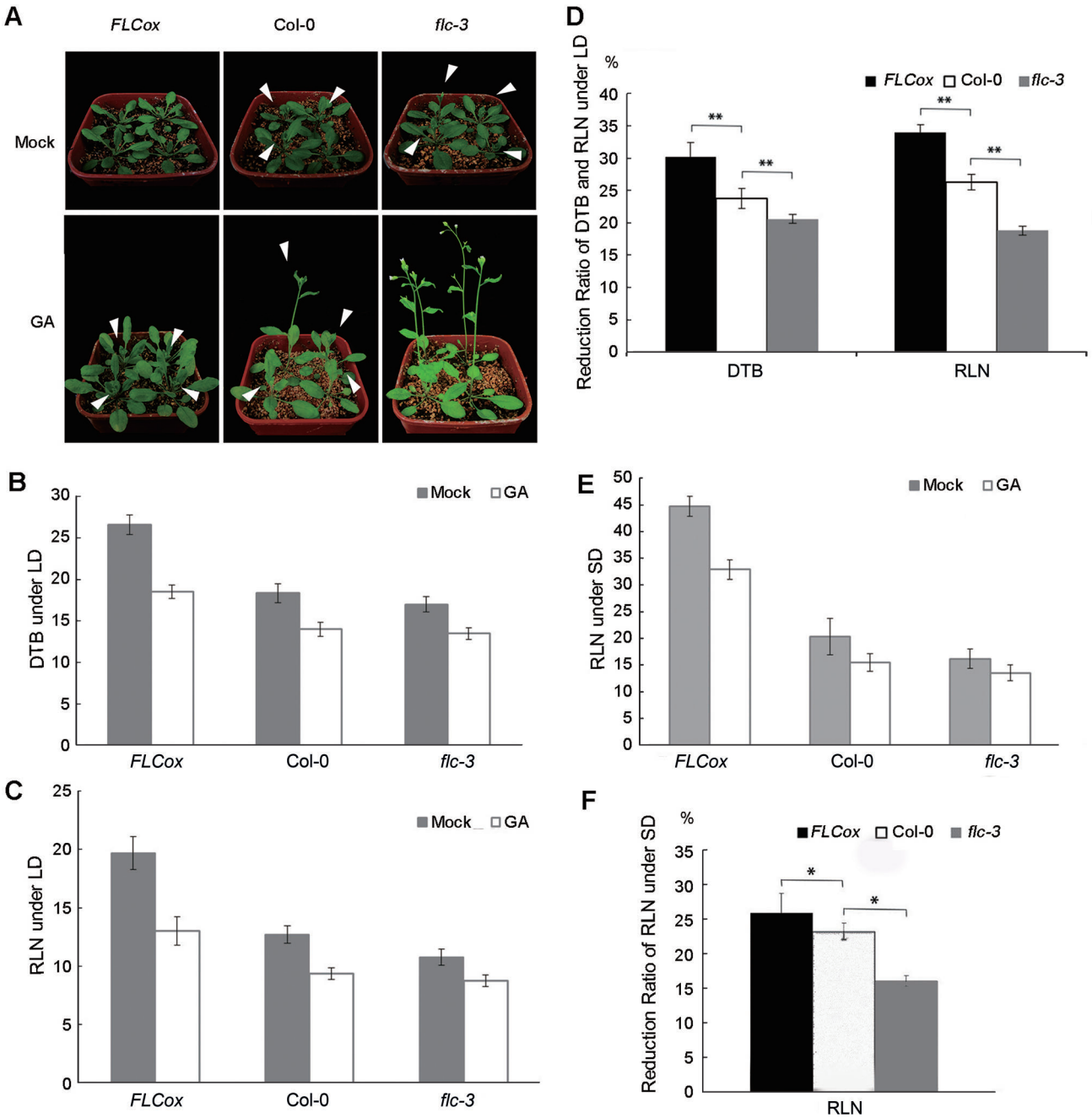
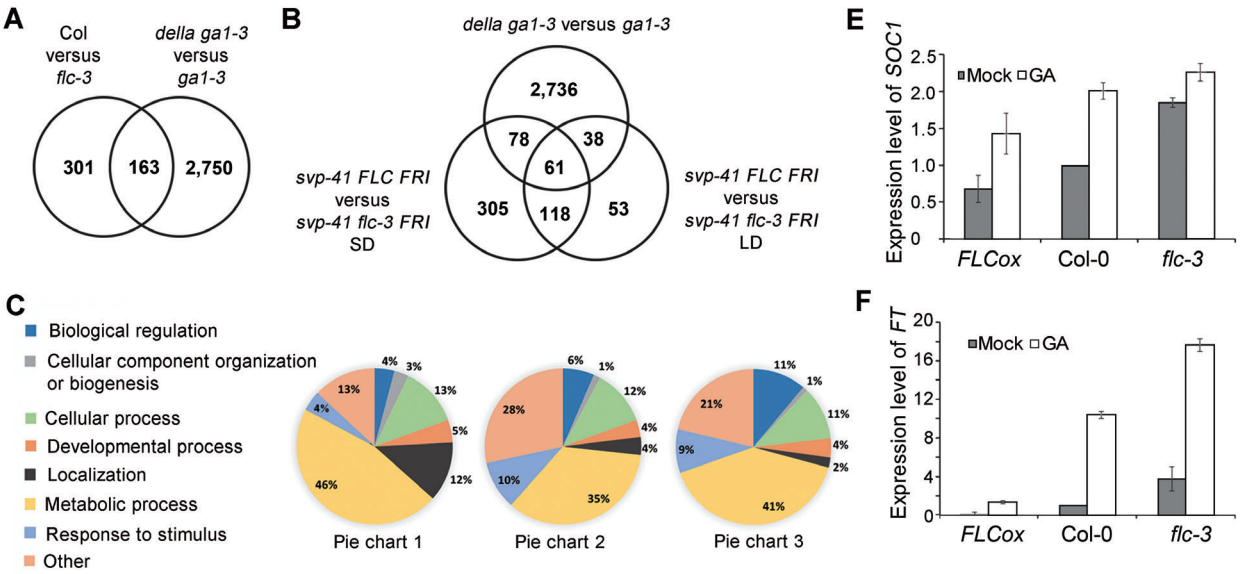


Figure 5. FLC partly mediates GA response in flowering time regulation

(A) Twenty-d-old *FLCox*, Col-0 and *flc-3* seedlings grown under long-day conditions (LD) treated with or without 50 μmol/L gibberellic acid (GA) every 2 d. The white arrows point at the position which shows the flower buds. **(B)** The days to bolt (DTB) of *FLCox*, Col-0 and *flc-3* plant treated with or without 50 μmol/L GA every 2 d under LD, error bars represent ± *SD*, $n = 35$. **(C)** The number of leaves upon bolting (RLN) of *flc-3*, Col-0 and *FLCox* plants treated with or without 50 μmol/L GA every 2 d under LD, error bars represent ± *SD*, $n = 35$. **(D)** Reduction ratio of DTB and RLN in response to GA under LD. The reduction ratio was calculated as (number of leaves/days (mock) - number of leaves/days (GA_3)/ number of leaves/days (mock)). **Student's *t*-test, $P < 0.01$. **(E)** RLN of *flc-3*, Col-0 and *FLCox* plants treated with or without 50 μmol/L GA every 2 d under short-day condition (SD), error bars represent ± *SD*, $n = 35$. **(F)** The reduction ratio of RLN in response to GA under SD. The reduction ratio was calculated as (number of leaves/days (mock) - number of leaves/days (GA_3)/ number of leaves/days (mock)). *Student's *t*-test, $P < 0.05$.

by ChIP-seq, 163 (35.1%) were also affected in *della ga1-3* compared to *ga1-3* (Figure 6A). To further verify that FLC and DELLAs regulate gene expression in a concerted manner, previously published microarray data between *svp-41* (*short vegetative phase-41*) *FLC FRI* and *svp-41 flc FRI* were compared to *della ga1-3* versus *ga1-3* (Cheminant et al. 2011; Mateos et al. 2015). Analysis of *svp-41 FLC FRI* and *svp-41 flc-3 FRI* grown for 2 weeks under short day conditions showed that 562 genes expressed differentially, among which 139 (24.7%) were also identified in the comparison of *della ga1-3* versus *ga1-3*. Using the same genotypes of seedlings grown for 2 weeks under short days and transferred to long days for 2 d, it showed that 270 genes were regulated by FLC, and 99 (36%) were also affect by DELLAs (Figure 6B). Gene Ontology analyses showed that the FLC/DELLA-co-regulated genes were enriched in metabolic process, cellular process, biological regulation, developmental process and response to stimulus (Figure 6C), revealing that FLC and DELLAs coordinately regulate a wide variety of genes in addition to flowering time genes, including *FT*, *SOC1*, *SEP3*, among others (Figure 6D).

To further confirm that FLC and DELLA cooperate to regulate flowering time at the molecular level, we detected the levels of *FT* and *SOC1* gene expression in *FLCox* or *flc* plants upon GA application. Thirty-day-old long day-grown plants with different treatments were collected. The expression levels of *FT* and *SOC1* were in good correlation with the flowering time phenotype, as evidenced by the levels of *FT* and *SOC1* mRNAs being markedly lower in *FLCox* than those in



D

Gene name	Bind by FLC	Regulated by FLC	Regulated by DELLA	Note	Reference
FT	YES	Down	Down	Promotes flowering	Hisamatsu and King, 2008;Cheminant et al., 2011;Deng et al., 2011;Porri et al., 2012; Yu et al., 2012
SOC1	YES	Down	Down	Controls flowering and is required for CO to promote flowering	Cheminant et al., 2011;Moon et al., 2003; Deng et al., 2011;Yu et al., 2012
SEP3	YES	Down	Down	SEP1/2/3 triple mutants show a conversion of petals and stamens to sepals	Cheminant et al., 2011;Deng et al., 2011; Mateos et al., 2015
AGL16	YES	Down	Down	MADS-box transcription factor, involve in flowering	Cheminant et al., 2011;Deng et al., 2011; Mateos et al., 2015
SVP	YES	NO	Down	Acts as a floral repressor, involve in thermosensory pathway	Cheminant et al., 2011;Deng et al., 2011
FLP1	YES	-	Down	Function unknown and is similar to flower promoting factor 1	Cheminant et al., 2011;Deng et al., 2011
VEL1	YES	-	Down	Involved in epigenetic silencing of the FLC	Cheminant et al., 2011;Deng et al., 2011
DDF1	YES	Up	Up	Overexpression of this gene results in delayed flowering and dwarfism, reduction of gibberellic acid biosynthesis, and increased tolerance to high levels of salt	Mateos et al., 2015
PRE1	-	Up	Up	Required for appropriate regulation of flowering time and is directly and negatively regulated by AP3 and PI in petals	Mateos et al., 2015

Figure 6. FLC and DELLAs co-regulate a large subset of genes

(A) Venn diagram from analyzing previously published ChIP-seq data showing the significant overlap between genes that are bound by FLC in Col versus *flc-3* and differentially expressed in *della ga1-3* versus *ga1-3*. **(B)** Venn diagram from analyzing previously published microarray data showing the overlap between sets of genes differentially expressed in *della ga1-3* versus *ga1-3*, *svp-41 FLC FRI* versus *svp-41 flc-3 FRI* grown for 2 weeks under short day conditions (SD), and *svp-41 FLC FRI* versus *svp-41 flc-3 FRI* grown for 2 weeks under SD and transferred to long day conditions (LD) for 2 d. **(C)** Gene Ontology analysis of biological processes represented by DELLA regulated and FLC bound gene (pie chart 1), by DELLA and FLC co-regulated gene under SD (pie chart 2), by DELLA and FLC co-regulated gene under LD (pie chart 3). **(D)** Flowering-related genes co-regulated by DELLA and FLC. **(E)** Expression of *SOC1* in 30-d-old seedlings treated with or without GA under LD, error bars represent ± *SD*, $n = 3$. **(F)** Expression of *FT* in 30-d-old seedlings treated with or without GA under LD, error bars represent ± *SD*, $n = 3$.

flc-3 and Col-0 (Figure 6E,F). These genomic data thus provide direct evidence to support the concerted action of FLC and DELLAs in regulating genome-wide gene expression, including several known flowering time genes.

DISCUSSION

Plants integrate multiple flowering pathways via FLC-DELLAs interaction

Intensive molecular and genetic studies have identified five flowering pathways in *Arabidopsis*, including photoperiod, vernalization, GA, aging and autonomous pathways (Amasino, 2010; Srikanth and Schmid, 2011). Elucidating the integrating mechanism of these pathways is vital for the understanding of how plants flower in response to diverse developmental and environmental signals. Previous studies integrated FLC-mediated vernalization and autonomous pathways with an FT-mediated photoperiod pathway as *FT* is a direct target gene of FLC that inactivates its transcription (Searle et al. 2006). GA and aging pathways are also interconnected by the interaction between DELLAs and SPL (Yu et al. 2012). Recent research places GATA transcription factors GNC and GNL downstream of GA signaling and upstream of SOC1, which may connect the photoperiod pathway with the GA signaling pathway (Richter et al. 2010; Richter et al. 2013). It was reported that vernalization and GA pathways converge at regulating *SOC1* expression, although the detailed molecular mechanism remains unknown (Moon et al. 2003). In this study, we revealed that vernalization/autonomous and GA pathways are integrated through a direct interaction between FLC and DELLAs, and such interaction is critical for robustly repressing *SOC1* and *FT* gene expression (Figure 7A). Therefore, GA pathway induces floral transition in at least three parallel pathways, in which DELLA proteins interact with FLC, PIF-GNC/GNL or SPL transcription factors with an opposite effect: DELLAs enhance FLC activity but repress PIF-GNC/GNL or SPL function (Figure 7A).

Our results demonstrate that DELLAs binding to FLC enhances transcriptional repression of FLC on *FT* and *SOC1* genes, and consequently delays the floral transition. We propose a model to illustrate how FLC and DELLA coordinate to regulate plant flowering (Figure 7B). In brief, DELLAs and FLC directly interact with each other and probably function in a large complex, in line with the previous finding that FLC exists in an approximately 800 kDa complex (Helliwell et al. 2006). Such a large complex may be required for a full inhibitory capacity to repress the target gene expression. GA

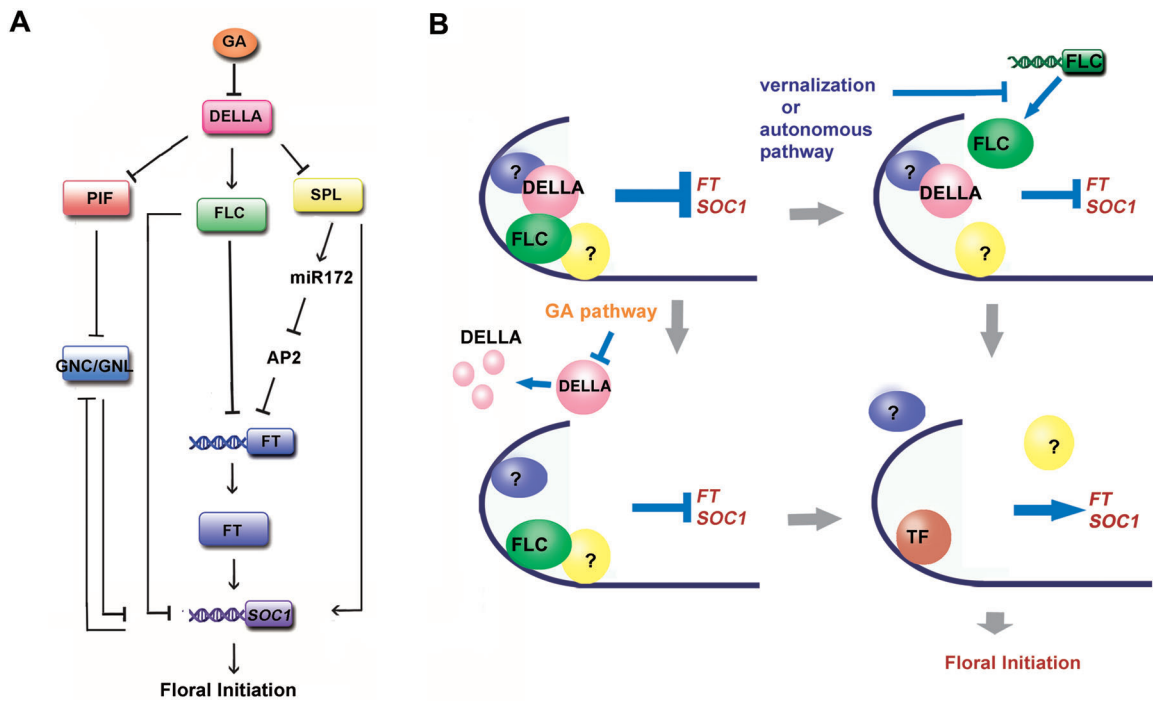


Figure 7. Model of the interaction of FLC and DELLA in control of flowering time

(A) GA pathway induces floral transition in at least three parallel pathways. In one pathway, DELLAs binding to FLC enhances transcriptional repression of FLC on *FT* and *SOC1* genes, while in another pathway, DELLAs directly interact with SPL and inhibit its transcriptional activities to downstream target genes. In addition, DELLAs indirectly regulate *SOC1* through the GATAs GNC and GNL. **(B)** A proposed model to illustrate how FLC and DELLA interact to regulate plant flowering. DELLAs and FLC directly interact with each other and probably function in a large complex to repress the target gene expression. GA induces the rapid degradation of DELLAs by 26S proteasome, resulting in the reduction (but not elimination) of the inhibition effect of the complex. Vernalization through a long period of cold exposure epigenetically silences *FLC* gene expression. In the absence of FLC, the complex losses its functional core and repressive capacity, thereby flowering-promoting genes are expressed and plants turn to flower.

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A new regulatory mechanism on FLC and a novel mode of DELLA action

FLC, encoding a MADS-box transcription factor (Michaels and Amasino 1999), acts as a strong repressor of floral transition by direct binding to the promoters of several flowering time genes (*SOC1* and *FT*) and repressing their transcription. Epigenetic regulation of *FLC* transcription by proteins and long noncoding RNAs has been extensively studied (Bastow et al. 2004; Sung and Amasino 2004; Margueron et al. 2005; Sung et al. 2006; Schmitz and Amasino 2007; Choi et al. 2011; Jeon and Kim 2011; Kim and Sung 2012). In this study, we demonstrate that FLC is also regulated post-transcriptionally. DELLAs interact with FLC and enhance the transcription inhibition ability of FLC, which may provide new insight into the FLC-mediated floral transition.

Although considerable progress has been made in illustrating the molecular basis of DELLAs' functions, it is still far from having a complete picture about different action modes of DELLAs. Recent studies revealed the existence of other regulatory mechanisms underlying DELLA-regulated plant growth and development processes. For example, DELLAs function as a transcriptional coactivator through interaction with other transcription factor(s) (Lim et al. 2013; Fukazawa et al. 2014; Yoshida et al. 2014). To add another layer of action mode, our study suggests that DELLAs may also act as co-repressors to suppress gene transcription by interacting with a negative transcription factor, in this case, FLC. This interaction enhances the transcription repression ability of FLC, although the detailed mechanism needs to be further investigated.

MATERIALS AND METHODS

Plant materials and growth condition

flc-3 (Michaels and Amasino 1999), *della* (*rga-2 gai-t6 rgl1-1 rgl2-1 rgl3-1*) mutants and *TAP-RGA* transgenic plants (Feng et al. 2008) were previously described. *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild type (WT) control. To construct transgenic plants constitutively expressing *FLC* (*FLCox* for short), *FLC* cDNA was amplified and digested by XbaI and BamHI, inserted into pFPZPY122 vector containing triple Flag-tag, and then introduced into Agrobacterium tumefaciens strain GV3101, and further transferred into Col-0 or *TAP-RGA* plants through the floral dipping method (Clough and Bent 1998).

Arabidopsis thaliana plants and *Nicotiana benthamiana* were grown at 22 °C in long days (16 h of light followed by 8 h darkness) or short days (8 h of light followed by 16 h darkness).

Chemical solutions

All chemicals were from Sigma-Aldrich, and prepared as stock solutions. Dimethyl sulfoxide was used to dissolve MG132 (10 mmol/L). One hundred percent ethanol was used to dissolve GA3 (71.43 mmol/L), 10 μmol/L MG132 for Co-IP and 50 μmol/L GA3 for seedling treatment.

Flowering time measurement and gene expression analysis

Surface-sterilized seeds were plated on Murashige and Skoog (MS) medium (4.3 g/L MS basal salts (PhytoTech, Inc.), 1% sucrose (Sigma-Aldrich, Inc.), pH 5.7 and 8 g/L agar (Sigma-Aldrich, Inc.)) and imbibed for 4 d at 4 °C. The seedlings were transferred into soil on the 5th d after exposing to light. Ten μmol/L GA_3 or mock was sprayed to the seedlings once every 2 d. Flowering time was measured by counting the numbers of rosette leaves and the days to bolt. Each experiment used more than 35 seedlings. All the plant materials were obtained on the zeitgeber time 16 h.

Total RNA was extracted with TRIzol reagent (Invitrogen, Inc.) and treated with DNase I (Promega, Inc.). A total of 2 μg RNA was added into the Moloney murine leukemia virus reverse transcription system (Promega, Inc.). Real-time PCR was performed in the LightCycler 480 Real-Time PCR system (Roche, Inc.) using SYBR Green Mix (Takara, Inc.). All the primers used in this study are listed in the Table S1 online. Three biological replicates were conducted for each experiment.

Yeast two-hybrid assay

Plasmids were transformed into yeast strain AH109 following the Match-marker user's manual protocol (Clontech, Inc.). Single fresh colonies of independent transformants were selected from SD/-Leu-Trp plates medium (Clontech, Inc.) and subsequently grown on SD/-Leu-Trp-His-Ade plates medium (Clontech, Inc.) to test interactions. All the primers used in this study are summarized in Table S1. Experiments were biologically repeated at least three times.

Western blot assays

Plant tissue was ground in liquid nitrogen. Protein was extracted in buffer (50 mmol/L Tris-Cl (Sigma-Aldrich, Inc.), pH 7.5, 150 mmol/L NaCl (Sigma-Aldrich, Inc.), 1 mmol/L ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Inc.), 0.25% Triton X-100 (Amresco, Inc.), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Solarbio, Inc.) and 1× protease inhibitor cocktail (Roche, Inc.)).

Yeast was collected into a 1.5 mL tube, and 0.5 volume 5× sodium dodecylsulfate (SDS) loading buffer (0.6 mL 1M Tris-cl (pH6.8), 2 mL 10% SDS (Sigma-Aldrich, Inc.), 0.5 mL β-mercaptoethanol (Amresco, Inc.), 1 mL 1% bromophenol blue (GE, Inc.) and 0.9 mL distilled water) was added. Thirty microliters acid-washed glass beads (Sigma) per 60 μL yeast cells were used for a thorough vortex. Yeast was repeatedly frozen-thawed for 3-8 times.

Anti-GST antibody (Sigma-Aldrich, Inc.), anti-HIS antibody (Sigma-Aldrich, Inc.), anti-FLAG antibody (Sigma-Aldrich, Inc.), anti-HA antibody (Abgent, Inc.) and anti-Myc antibody (Abgent, Inc.) were diluted 5,000-fold.

Pull-down assay

Plasmids harboring *GST-RGA* (RGA is cloned in pGEX-5X-1 (GE Healthcare, Inc.)) or *HIS-FLC* (pET-16b (Novagen, Inc.)) were

transformed into BL21 (DE3)-competent cells. Protein expression was induced by 0.1 mmol/L isopropyl-beta-thiogalactopyranoside and fusion proteins were prepared following the manufacturer's instructions.

In vitro expressed and purified HIS-FLC was incubated with Ni-NTA beads (QIAGEN, Inc.) in pull-down buffer (50 mmol/L Tris-Cl, pH 8.0, 150 mmol/L NaCl, 10% glycerol, 0.5 mmol/L EDTA, 0.1% Triton X-100, 5 mmol/L mercaptoethanol and 1× protease inhibitor cocktail) for 4 h at 4 °C, and then GST-RGA was added and incubated together with HIS-FLC for another 3 h at 4 °C. Beads were collected by gentle centrifugation and washed five times by pull-down buffer. Proteins were eluted in protein extraction buffer and loaded on SDS polyacrylamide gel electrophoresis for western blot. Experiments were biologically repeated at least three times.

Bimolecular fluorescence complementation assay (BiFC)

pUC-SPYNE (nYFP) and pUC-SPYCE (cYFP) vectors were used to construct split YFP fusion proteins or as negative controls (Walter et al. 2004). Plasmids were transformed into protoplasts of *Arabidopsis thaliana* suspension cells as previously described (Miao and Jiang 2007). Fluorescence was visualized under a Nikon 80i fluorescence microscope. Experiments were biologically repeated at least two times.

Coimmunoprecipitation assay

Co-IP assays of the FLC-RGA proteins were performed with 8-d-old *FLCox/TAP-RGA* T2 seedlings grown under white light. Seedlings were treated with 10 μmol/L MG132 for 4 h prior to protein extraction. Immunoprecipitation of the FLC-3FLAG protein was performed at 4 °C for at least 4 h, using anti-FLAG M2-agarose beads (Sigma-Aldrich, Inc.) in a buffer containing 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.25% Triton X-100, 10 μmol/L MG132, 1 mmol/L PMSF, and 1× protease inhibitor cocktail (Roche, Inc.). FLC-3FLAG and RGA-MYC detection were performed with anti-FLAG and anti-MYC antibodies, respectively. Experiments were biologically repeated at least three times.

ChIP assay

ChIP was performed as described (Chen et al. 2009) using 2-week-old FLCox/TAP-RGA seedlings incubated in liquid MS supplemented with 50 μmol/L GA or mock 8 h. Mouse anti-MYC antibodies (Abgent, Inc.) or anti-FLAG (Sigma-Aldrich, Inc.) were added into sheared chromatin and incubated overnight at 4 °C to allow immunizing bound DNA fragments. DNA was pelleted by Recombinant Protein G-Sepharose 4B (Invitrogen, Inc.) and eluted to amplify the sequences neighboring the FLC binding sites in *SOC1* and *FT* using primer pairs listed in Table S1, and 3′-UTR primer pairs were used as control. Three biological replicates were conducted for each experiment.

Dual-luciferase report assays

SOC1 and *SEP3* 1.5 kilobases promoter regions were amplified from the *Arabidopsis* genomic DNA and cloned into pGreen II 0800-LUC as reporters (Hellens et al. 2005). The coding sequences of *RGA* and *FLC* were amplified and inserted into pGreen II 62-SK as effectors (Hellens et al. 2005). *Agrobacterium* strain GV3101 carrying the reporter plasmid and specific effectors was cultured to $OD_{600} = 0.5$ respectively, 1:2 combined in volume, incubated at room temperature without shaking for 3 h and infiltrated into *N. benthamiana* leaves. The dual-luciferase reporter system (Promega, Inc.) was used to analyze the transient expression in *N. benthamiana* 2 d after infiltration. The activities of firefly (*Photinus pyralis*) and *Renilla reniformis* luciferases were measured sequentially from a single sample on a GLO-MAX 20/20 luminometer (Promega, Inc.) The ratio of LUC to REN was calculated to indicate the final transcriptional activity. Four biological repeats were measured for each sample.

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AUTHOR CONTRIBUTIONS

M.L. performed most of the research and drafted the manuscript. M.M. and W.L. performed some expressions and analyses. Y.F. and X.Z. revised the manuscript. F.A. and H.G. designed the experiment, supervised the study and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Western blot detection of proteins which did not interact in yeast two hybrid

(A) Western blot determined the expression of protein FLC 58-93 aa, FLC-△MADS, RGA-M5, GAI-M5.Total protein was extracted from yeast strain FLC 58-93/RGA-M5, FLC 58-93/GAI-M5, FLC-△MADS/RGA-M5, FLC-△MADS/GAI-M5 grown on SD/-Leu-Trp medium. Yeast strain co-transferred AD/BD was used as negative control. **(B)** Western blot determined the expression of protein FLC 1-93 aa and RGA-M5△LHRI. Total protein was extracted from yeast strain FLC 1-93 aa/ RGA-M5△LHRI grown on SD/-Leu-Trp medium. Yeast strain co-transferred AD/BD was used as negative control.

Figure S2. Western blot analysis of FLC overexpression in transgenic plants

(A) Western blot determined expression of FLC-flag fusion protein. Total protein was extracted from 8-d-old *TAP-RGA* and *FLCox/TAP-RGA* transgenic lines for immunoblot assay with anti-FLAG antibody. **(B)** Western blot determined expression of FLC-flag fusion protein. Total protein was extracted from 8-d-old Col-0 and *FLCox* transgenic plants for immunoblot assay with anti-FLAG antibody.

Table S1. The primers used in this paper